ASBMT
American Society for Blood and Marrow Transplantation

Pharmacologic Expansion of Donor-Derived, Naturally Occurring CD4$^+$Foxp3$^+$ Regulatory T Cells Reduces Acute Graft-versus-Host Disease Lethality Without Abrogating the Graft-versus-Leukemia Effect in Murine Models

Omar Duramad,[1] Amy Laysang,[1] Jun Li,[1] Yasuyuki Ishii,[2] Reiko Namikawa[1]

Adoptive transfer of regulatory T cells (Tregs) prevents graft-versus-host disease (GVHD) in mouse models, indicating a pivotal role for Tregs in controlling GVHD. The present study demonstrates the efficacy of Tregs pharmacologically induced in vivo in GVHD prevention. A single i.v. administration of a liposomal formulation of α-galactosylceramide (RGI-2001) at the time of allogeneic bone marrow transplantation with spleen cells significantly prolonged the survival of mice experiencing lethal acute GVHD. RGI-2001 expanded donor-derived CD4$^+$Foxp3$^+$ Tregs in the spleen, lymph nodes, and bone marrow in a dose-dependent manner. On day 15 posttransplantation, the spleens of mice treated with RGI-2001 (1 μg/kg) contained 5-fold higher percentages or 10-fold higher numbers of CD4$^+$Foxp3$^+$ Tregs compared with the spleens of untreated mice. Host-specific immunosuppression was introduced in treated mice, whereas the responsiveness to third-party alloantigens and leukemia cells was maintained. Using Foxp3:GFP reporter mice as donors, it was clearly shown that RGI-2001 expanded the pre-existing naturally occurring Tregs (nTregs) in donor spleen cells. Finally, RGI-2001 synergized with a subtherapeutic dose of rapamycin in nTreg expansion and further prolonged survival. Our results provide the first demonstration of the efficacy of nTregs pharmacologically expanded in vivo in preventing acute GVHD without abrogation of the beneficial graft-versus-leukemia effect.

Biol Blood Marrow Transplant 17: 1154-1168 (2011)

KEY WORDS: Treg cells, Bone marrow transplantation, Tolerance induction, GVHD, GVL

INTRODUCTION

The CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) are considered the key players in maintaining peripheral tolerance [1,2]. Animal studies have demonstrated that these cells prevent or ameliorate various T cell–mediated diseases, including autoimmune diseases and transplantation, by restoring immune tolerance to self-antigens as well as to alloantigens [3]. Studies in both humans and mice have demonstrated the pivotal role of Tregs in controlling graft-versus-host disease (GVHD) [4]. The development of Treg-based therapeutic modalities has been hampered, however, due in part to the paucity of this cell population. Although conditions to expand Tregs in vitro have been established [5,6], translating Tregs into the clinical setting has been hindered by technical challenges, including isolation, large-scale expansion, and regulatory requirements [7]. The discovery of molecules that can efficiently expand functional Tregs in vivo would contribute to the development of novel therapies for the treatment of GVHD as well as other immune disorders. Various strategies to activate and expand Tregs in situ are emerging [8].

KRN7000 is a synthetic derivative of α-galactosylceramide, a glycolipid originally discovered from a marine sponge [9]. It serves as a ligand for the CD1d molecule, expressed on antigen-presenting cells [10,11]. The CD1d molecule is a nonpolymorphic, major histocompatibility complex (MHC) class I–like antigen-presenting molecule with an antigen-binding groove adapted for the presentation of lipid antigens [12,13]. When KRN7000 is presented by CD1d molecules expressed on various cell types, including

From the [1]Research and Development, REGiMMUNE Inc, Santa Clara, California; and [2]Laboratory for Vaccine Design, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan.

Financial disclosure: See Acknowledgments on page 1167.

Correspondence and reprint requests: Reiko Namikawa, MD, PhD, REGiMMUNE Inc, 4800 Great America Parkway, Suite 222, Santa Clara, CA 95054 (e-mail: reiko@regimmune.com).

Received July 21, 2010; accepted November 18, 2010

1083-8791/$36.00
doi:10.1016/j.bbmt.2010.11.022

dendritic cells (DCs), macrophages, and B cells, it is recognized by an invariant T cell receptor (TCR) expressed on invariant natural killer T (iNKT) cells, and activates iNKT cells in a CD1d-restricted manner [14]. CD1d-restricted iNKT cell activation results in a rapid release of large amounts of both Th1 and Th2 cytokines, a unique property that distinguishes iNKT cells from conventional T cells and suggests their important immunoregulatory roles in both innate and adaptive immunity.

Whereas KRN7000, given in an aqueous form, has been shown to act as an immunostimulating agent, a liposomal formulation of KRN7000 was found to induce antigen-specific immune suppression or tolerance [15,16]. Previous studies have suggested that differential cell targeting by liposomal KRN7000 results in different immunomodulatory responses. In brief, aqueous KRN7000 is presented mainly by DCs, thereby stimulating iNKT cells in the presence of interleukin (IL)-12 secreted by DCs. In contrast, the interaction of iNKT cells with KRN7000 presented on B cells induces production of IL-10 from both iNKT cells and B cells, resulting in expansion of tolerogenic DCs [15], which subsequently induces the generation of antigen-specific $Foxp3^+CD4^+CD25^{high}$ Tregs in the presence of a model antigen, ovalbumin [15,16]. It is conceivable that the lipid composition of liposomes enhances the uptake of the liposomal KRN7000 by B cells, thereby skewing immune responses toward the tolerogenic direction.

In the present study, we investigated whether a liposomal KRN7000 (RGI-2001) can induce alloantigen-specific tolerance through Treg induction. Specifically, we evaluated the efficacy of RGI-2001 in a murine acute GVHD model, and found that a single injection of RGI-2001 significantly prolonged the survival of mice. Enhanced expansion of donor-derived $CD4^+Foxp3^+$ Tregs was seen to be the key underlying mechanism. Host-alloantigen specific immune suppression was induced early after bone marrow transplantation (BMT), but responses to third-party alloantigens or leukemia cells were not abrogated. Our results suggest the possibility of developing novel therapies for the prevention of GVHD by pharmacologically exploiting intrinsic immunoregulatory pathways for induction and maintenance of tolerance.

MATERIALS AND METHODS

Mice

The 8- to 10-week-old female BALB/c (H-2^d), C57BL/6J (H-2^b), FVB (H-2^q), C3H (H-2^k), and Foxp3:GFP reporter mice [17] (C57BL/6 background) were obtained from Jackson Laboratory (Sacramento, CA, or Bar Harbor, ME). All experiments were approved by an internal and external Committee on the Use and Care of Laboratory Animals.

Treatments

RGI-2001 (KRN7000 embedded in a liposomal bilayer) and the control liposomal formulation were prepared at REGiMMUNE. The doses were expressed based on the dose of KRN7000 and calculated assuming a mouse body weight of 20 g. RGI-2001 and the control formulation were diluted in 1× Hank's buffered saline solution for i.v. injection. Rapamycin (RAPA) from *Streptomyces hygroscopicus* (R0395; Sigma-Aldrich, St Louis, MO) was dissolved in a 1% carboxymethylcellulose sodium salt solution (C5013; Sigma-Aldrich) and stored in the dark at 4°C. RAPA was administered intraperitoneally (i.p.).

Antibodies

The following fluorochrome-conjugated antibodies were used for flow cytometry analysis: CD4, CD25, TCR-β, H-2kd, and H-2kb (all from BD Pharmingen, San Diego, CA) and anti-Foxp3 antibody (eBioscience, San Diego, CA). iNKT cells were detected using a KRN7000-loaded CD1d:tetramer (Proimmune, Oxford, UK) as described previously [18]. Staining was performed in the presence of purified anti-CD16/32 (BD Pharmingen) at saturation to block unspecific staining.

Flow Cytometry

Cell samples were analyzed with a Calibur flow cytometer (BD Biosciences, San Jose, CA), and acquired data were analyzed using FloJo software (Treestar, Ashland, OR). The percentage of iNKT cells was indicated as the CD1d $tetramer^+$ cells in the total $TCR\text{-}\beta^+$ cells, and that of $CD4^+Foxp3^+$ Tregs was indicated as the $Foxp3^+$ population among the total $CD4^+$ cells. Single-cell suspensions from spleens of Foxp3:GFP reporter mice were sorted on a FACSAria cell sorter (BD Biosciences) for the $Foxp3{:}GFP^-$ population.

Cytokine Measurements

Serum samples were analyzed for cytokines using RodentMAP v 2.0 (Rules-Based Medicine, Austin, TX).

GVHD Models and Cell Isolation

Lethally irradiated Balb/c mice were used as recipients, and C57BL/6, FVB, or Foxp3:GFP reporter mice were used as donors. Recipient mice were irradiated with 800 cGy at Stanford Research Institute using a Pantak X-ray energy source on the day of transplantation. Dynal Mouse Pan T (Thy1.2) beads (Invitrogen, Carlsbad, CA) were used to remove T cells from the donor bone marrow cells (BMCs). Depletion of T cells was verified by fluorescence-activated cell

sorter (FACS) analysis using TCR-β staining, which was routinely >90% for C57BL/6 and >97% for Foxp3:GFP reporter mice. A total of 5×10^6 T cell–depleted (TCD) BMCs were infused via a tail vein with 2.5×10^6 donor whole spleen cells (WSCs). RGI-2001 was administered i.v. via another tail vein approximately 30 minutes after donor cell infusion. The day of BMT was considered day 0. Mice were maintained under sterile conditions with autoclaved cages, bedding, and water supplemented with Polymixin B and were observed daily for survival.

Mixed Lymphocyte Reaction

WSCs isolated from recipient mice were used as responder cells. Allogeneic WSCs, treated with mitomycin C (50 μg/mL; Sigma-Aldrich) and depleted for T cells as described above, were used as stimulator cells. Cultures were evaluated in triplicate in 96-well round-bottomed plates in a total volume of 200 μL. Cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 10 mM Hepes (pH 7.2; Invitrogen), 1× L-glutamine (2 mM), 1× Na pyruvate (1 μM) (Invitrogen), 1× penicillin-streptomycin (Invitrogen), and 0.5× antibiotic/mycotic (Invitrogen). The optimal coculture condition was established at a 10:1 responder:stimulator ratio. Cell proliferation was analyzed on day 3 or day 4 by a colorimetric assay using alamarBlue (Serotec, Raleigh, NC).

Tumor Model

The in vivo adapted A20-*luc/yfp* cell line (Balb/c origin), transduced with the yellow fluorescent protein (YFP)/luciferase genes [19], was a generous gift from Dr. Robert Negrin, Stanford University Medical Center. A total of 5×10^4 A20-*luc/yfp* cells were administered i.v. into irradiated Balb/c recipients approximately 60 minutes before the infusion of TCD BMCs and WSCs. Mice were examined daily. The presence of tumor cells was evaluated macroscopically at the time of necropsy and analyzed by FACS based on the YFP signal detectable by the FL1 laser [20].

Statistical Analysis

Statistical data were analyzed using the Student's *t* test (unpaired, two-tailed). GVHD Kaplan-Meier survival curves were analyzed using the log-rank test. A P value $<.05$ was considered statistically significant.

RESULTS

RGI-2001 Induces Cytokine Production and iNKT Cell Expansion In Vivo

The activities of RGI-2001 in cytokine production and iNKT cell expansion were investigated in Balb/c and C57BL/6 mice. Administration of varying doses of RGI-2001 induced elevated serum levels of IL-2, IL-4, and interferon (IFN)-γ in a dose-dependent manner (Figure S1A-C). The peak levels of IL-2 and IL-4 were measured at 2 hours postinjection, and that of IFN-γ was seen at 16-20 hours postinjection. RGI-2001 induced expansion of iNKT cells in a dose-dependent manner, reaching a maximum 4- to 6-fold expansion (Figure S1D). These observations are consistent with findings reported previously with an aqueous form of KRN7000 [21]. In general, induction of cytokines and iNKT cells was observed at doses of ≥ 0.01 μg/kg. Treatment with the control formulation at a lipid dose equivalent to 10 μg/kg of RGI-2001 did not induce cytokines or expand iNKT cells (data not shown).

RGI-2001 Treatment Prolongs Mouse Survival in an Acute GVHD Model

The efficacy of RGI-2001 in attenuating the course of acute GVHD was evaluated using a murine lethal GVHD model with complete MHC-mismatched BMT (C57BL/6 →Balb/c). Varying doses (0.001, 0.01, 0.1, 1, 10, and 100 μg/kg) of RGI-2001 or the liposomal control containing a lipid dose equivalent to that of 10 μg/kg of RGI-2001 were administered i.v. immediately after TCD BMC + WSC transfusion, and survival was followed over 100 days. Efficacy was evaluated in at least two experiments for each dose, and the combined results of two representative experiments are shown in Figure 1. Typically, untreated mice died by day 40-50 in this model. The survival curve of mice that received the liposomal control was similar to that of untreated mice (Figure 1A). Statistically significant prolonged survival was seen in mice that received RGI-2001 at doses ≥0.1 μg/kg, and 30%-60% of mice in these treatment groups survived for >100 days (Figure 1A and B). Although RGI-2001 treatment significantly prolonged survival and reduced GVHD mortality, the mice developed substantial clinical manifestations of acute GVHD, including weight loss, hunched posture, diarrhea, and ruffled fur. Clinical appearance improved in survivors, and long-term (>100 days) survivors were nearly free of symptoms. Although a modest but significant increase in survival was observed at a dose of 0.01 μg/kg ($P = .0369$), no mice survived for >100 days in this group (Figure 1B). No significant effect was noted in the mice that received 0.001 μg/kg of RGI-2001 (data not shown). Collectively, the efficacious dose of RGI-2001 was determined to be ≥0.1 μg/kg. Survival curves at doses of 0.1, 1, 10, and 100 μg/kg were comparable overall, and no trend toward dose-related differences was noted within the effective dose range. Thus, 1 μg/kg was selected as the standard dose used in the rest of this study, unless specified otherwise.

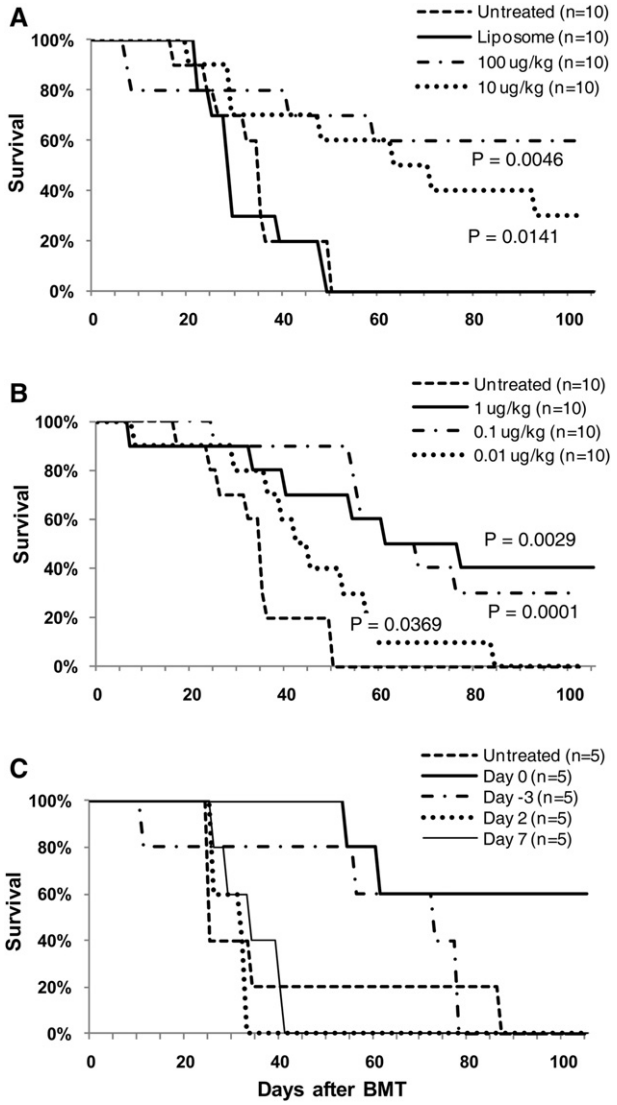

Figure 1. Effects of RGI-2001 treatment on acute GVHD mortality. A and B, Effects of varying doses of RGI-2001 and the liposomal control on the survival of lethally irradiated Balb/c mice reconstituted with 5×10^6 TCD BMCs and 2.5×10^6 WSCs of C57BL/6 (C57BL/6 → Balb/c). RGI-2001 was administered i.v. 30 minutes after the infusion of TCD BMCs and WSCs. The liposome control contained a lipid dose equivalent to 10 μg/kg of RGI-2001. For each dose, results of two representative experiments were combined. *P* values were calculated versus untreated controls. C, The impact of treatment schedule on efficacy. Results of a representative experiment from 4 experiments are shown. Survival was compared in mice receiving RGI-2001 (1 μg/kg i.v.) on day 0, day -3, day 2, or day 7 and untreated mice (n = 5 for each group). The highest efficacy was achieved by RGI-2001 administered on day 0, whereas efficacy was abrogated with delayed administration (day 2 or day 7).

The effect of the treatment timing was evaluated by administering RGI-2001 (1 μg/kg) before (day -3), concurrent with (day 0) or after (day 2 or day 7) BMT. Representative results are shown in Figure 1C. Robust efficacy was reproducibly observed in mice treated on day 0, modest efficacy was seen in mice treated on day -3, and no efficacy was seen in mice treated on day 2 or day 7. These results were reproduced in 4 independent experiments. It was concluded that administration on day 0 (at the time of BMT) was the most effective treatment schedule.

The levels of various serum proteins were investigated on day 6 post-BMT (Table 1). Statistically significant decreases were noted in the levels of IFN-γ and tumor necrosis factor-α in mice treated with RGI-2001 (1 μg/kg) compared with untreated mice. In addition, levels of acute-phase proteins (C-reactive protein and haptoglobin) and chemokines (IP-10, macrophage inflammatory protein [MIP]-1β, and MIP-1γ) were significantly reduced in RGI-2001–treated mice, suggesting decreased severity of inflammation and leukocyte trafficking in these mice. No differences were noted in the levels of other cytokines, including IL-6 and IL-10. IL-2, IL-12p70, and IL-17 were below detection levels in both groups.

RGI-2001 Promotes Expansion of the Donor-Derived Treg Compartment Early after BMT

We hypothesized that RGI-2001 treatment might reduce the severity of GVHD by inducing transplantation tolerance through the induction of Tregs, and thus investigated Treg recovery after BMT. On day 15 post-BMT, cells from spleen; mesenteric lymph nodes (mLNs); peripheral lymph nodes (pLNs), including inguinal and axillary nodes; bone marrow (BM); and peripheral blood (PB) were analyzed for $CD4^+$ $Foxp3^+$ Tregs. First, it was confirmed that all cells (including $CD4^+$ cells) in these organs were reconstituted with H2-Kb^+ donor-derived cells in both RGI-2001–treated and untreated mice (Figure 2A, upper panels). Staining for Foxp3 revealed marked increases in $Foxp3^+$ cells within the $CD4^+$ cell population in RGI-2001–treated mice compared with untreated mice (Figure 2A, bottom panels). Higher percentages of $CD4^+Foxp3^+$ Tregs were consistently found in the tissues examined. This finding was highly reproducible and confirmed to be significant in 4 independent experiments. Combined data (Figure 2B, left) demonstrated that 3- to 5-fold increases in the percentage of $CD4^+Foxp3^+$ cells were induced systemically by RGI-2001 treatment by day 15 post-BMT. Differences were statistically highly significant in all organs investigated. The absolute number of $CD4^+Foxp3^+$ Tregs calculated in the spleen samples was $0.8 \pm 0.5 \times 10^5$ cells in untreated mice (n = 5) and $7.5 \pm 1.3 \times 10^5$ cells in RGI-2001–treated mice (n = 5). The ~10-fold increase in the number of Tregs found in treated mice is highly statistically significant ($P < .0001$). Higher percentages of Tregs were maintained in RGI-2001–treated mice compared with untreated mice on day 30 post-BMT (Figure 2B, right); however, a highly statistically significant difference ($P < .005$) was found only in the spleen. The percentage of Tregs in mLNs and pLNs in RGI-2001–treated

Table 1. Serum Protein Levels on Day 6 Post-BMT

	IFN-γ, pg/mL	TNF-α, pg/mL	CRP, μg/mL	Haptoglobin, μg/mL	IP-10, pg/mL	MIP-1β, pg/mL	MIP-1γ, ng/mL
Untreated	395 ± 66	207 ± 17	3.96 ± 0.39	253 ± 27	3158 ± 271	1545 ± 177	109 ± 4
RGI-2001	181 ± 86*	149 ± 29*	2.81 ± 0.25**	79 ± 45**	1448 ± 341**	784 ± 147**	87 ± 12*

CRP indicates C-reactive protein; TNF, tumor necrosis factor.
Sera from untreated and RGI-2001–treated (1 μg/kg) mice (n = 4 for each group) were collected on day 6 post-BMT and analyzed using RodentMAP v 2.0 (Rules-Based Medicine, Austin, TX).
*P <.05.
**P <.005 versus untreated mice.

mice decreased compared with values measured on day 15 post-BMT. In addition, as the earlier time point, Tregs in the spleen were investigated on day 6 post-BMT. The percentage of $CD4^+Foxp3^+$ Tregs was 1.7% ± 0.5% in untreated mice (n = 4) and 5.6% ± 1.5% in RGI-2001–treated mice (n = 4). The increases were highly statistically significant compared with untreated mice (P = .0026), although the level of Tregs in the treated mice was lower than that measured on day 15 (14.8% ± 7.6%). Taken together, these results demonstrate that RGI-2001 treatment induced expansion of Tregs early after BMT, during the GVHD initiation period, and that Tregs reached their highest level in the secondary lymphoid organs around day 15.

Host Alloantigen-Specific Immune Suppression Is Induced in RGI-2001–Treated Mice by Day 15 Post-BMT

The immunologic status of RGI-2001–treated mice was analyzed by an in vitro mixed lymphocyte reaction (MLR) assay. WSCs from RGI-2001–treated (1 μg/kg) and untreated Balb/c mice reconstituted with C57BL/6 BMC were harvested on day 15 post-BMT and then cocultured with MMC Mitomycin C-treated TCD stimulator cells derived from Balb/c (host) or C3H (third party) mice (Figure 2C). Marked suppression in response to Balb/c stimulators was observed in cultures with cells from RGI-2001–treated mice compared with those with cells from untreated controls. In contrast, no suppression was observed in responses against the third-party strain C3H. The percentage of $CD4^+Foxp3^+$ Tregs in the spleen cells was 2.57% ± 0.82% in the untreated mice (n = 5) and 18.8% ± 5.2% in the RGI-2001–treated mice (n = 5). This finding suggests that Tregs generated in RGI-2001–treated mice may be responsible for the host-specific MLR suppression, and that these Tregs may be functional and enriched for those Tregs reactive to host alloantigens.

RGI-2001 Induces Treg Expansion in a Dose-Dependent Manner

We investigated the effects of varying doses of RGI-2001, ranging from 0.001 to 100 μg/kg, on the induction of Tregs. Mice were analyzed on day 15 post-BMT (Figure 3). The combined results of 6 experiments demonstrated clear dose-dependent increases in the percentage of $CD4^+Foxp3^+$ cells in both spleen (Figure 3A) and mLNs (Figure 3B). The Treg levels in pLNs were significantly increased even at the lowest dose evaluated (Figure 3C). The total Treg cell counts in the spleen also increased in a dose-dependent manner (Figure 3D), with 13.1 ± 8.7 × 10^5 Tregs in mice treated with 100 μg/kg RGI-2001 compared with 0.3 ± 0.2 × 10^5 Tregs in untreated mice. Overall, statistically significant Treg induction was evident at doses ≥0.01 μg/kg. Our findings indicate that RGI-2001 promotes expansion of the donor-derived Tregs early after BMT in a dose-dependent manner.

Efficacy Is Reproduced in a GVHD Model with a Different MHC Combination

To confirm that the observed effects of RGI-2001 were not specific to the strains used in the foregoing experiments, efficacy was evaluated in a model involving a different strain combination, FVB (H2q)→ Balb/c (H2-Kd). RGI-2001 (1 μg/kg) significantly prolonged the survival of these mice (P = .029) (Figure 4A). T cell depletion in donor BMCs was not performed in this experiment, due to the Thy1-1 haplotype of FVB, which might explain the better outcome of untreated mice, with 60% survival at >100 days. Nevertheless, the results confirm that RGI-2001 treatment significantly improved survival of mice in a GVHD model involving a different MHC combination. The induction of Tregs by RGI-2001 was also confirmed in this model. On day 15 post-BMT, a higher percentage of $CD4^+Foxp3^+$ cells was detected in all organs in RGI-2001–treated mice (1 μg/kg), with statistically significant increases in spleen, pLNs, and PB (Figure 4B). The absolute Treg count in the spleen was 2.5-fold higher in the RGI-2001–treated group than in the untreated group, a statistically significant difference (P = .0301).

RGI-2001 Induces Expansion of Naturally Occurring Tregs

The origin of the Tregs induced by RGI-2001 was investigated using Foxp3:GFP reporter mice [17] (C57BL/6 background) as donors. Foxp3:GFP^+ pre-existing naturally occurring Tregs (nTregs) were

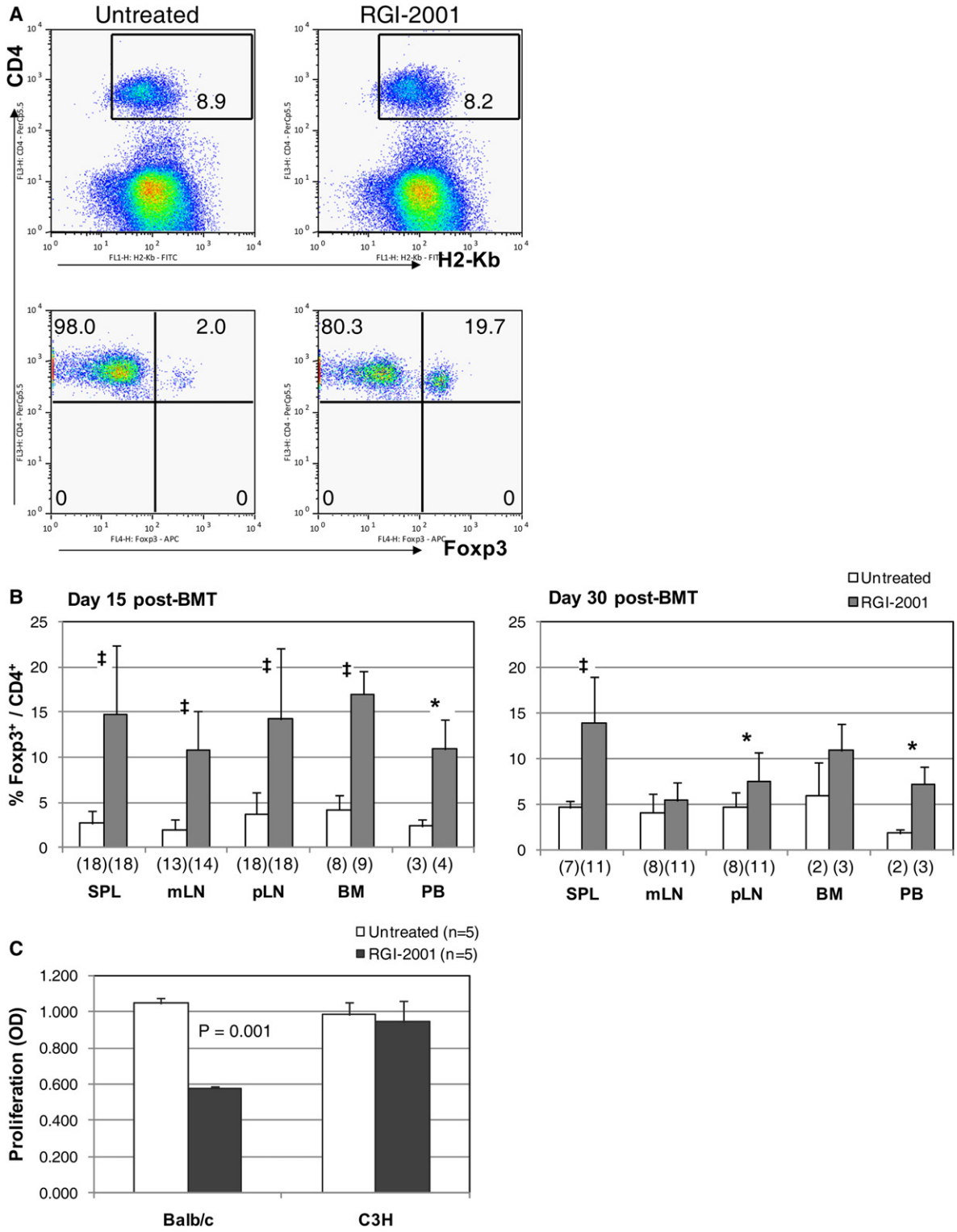

Figure 2. Early expansion of donor-derived $CD4^+Foxp3^+$ Tregs and induction of host alloantigen-specific immune suppression by RGI-2001. A and B, Recovery of $CD4^+Foxp3^+$ Tregs was investigated in untreated and RGI-2001–treated (1 μg/kg, day 0) mice. A, Representative dot plots of spleen cells analyzed on day 15 post-BMT. Upper panels: All $CD4^+$ cells were confirmed to be $H2\text{-}Kb^+$ donor-derived cells. Numbers indicate the percentage of $CD4^+$ cells in total spleen cells. Lower panels: Foxp3 expression in gated $CD4^+$ cells. Numbers in the upper-right quadrant indicate the percentages of $CD4^+Foxp3^+$ Tregs in total $CD4^+$ T cells. B, Mean ± standard deviation (SD) percentages of $CD4^+Foxp3^+$ cells in total $CD4^+$ cells in the spleen, mLNs, pLNs, BM, and PB analyzed on day 15 (left) and day 30 (right) post-BMT. The data combine results from 4 independent experiments; numbers in parentheses indicate the number of animals analyzed. *P* values versus untreated mice: $*P < .05$; $‡P < .005$. C, Spleen cells from Balb/c mice reconstituted with C57BL/6 TCD-BMC and WSC were harvested on day 15 post-BMT and subjected to MLR coculture stimulated with either MMC Mitomycin C-treated TCD Balb/c (host) or C3H (third party) spleen cells. The cultures were evaluated in triplicate. Mean ± SD values in mice treated with RGI-2001 (1 μg/kg) (n = 5) and in untreated mice (n = 5) are shown. The percentage of $CD4^+Foxp3^+$ Tregs in the spleen cells in this experiment was 2.57% ± 0.82% in the untreated mice and 18.8% ± 5.2% in the RGI-2001–treated mice. *P* value is versus untreated.

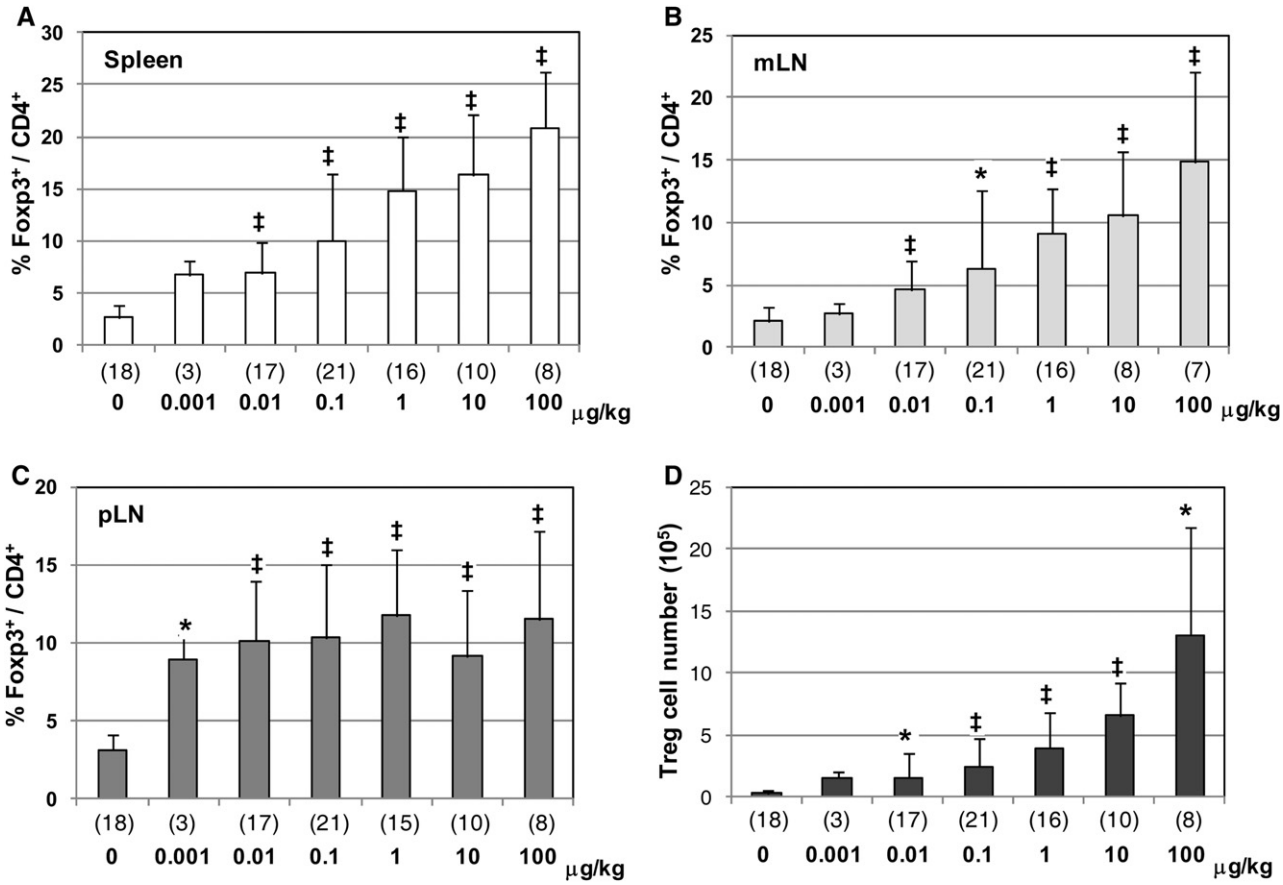

Figure 3. Dose-dependent induction of Tregs by RGI-2001 treatment. A, B, and C, Mean ± SD percentages of $CD4^+Foxp3^+$ cells in total $CD4^+$ cells in the spleen (A), mLNs (B), and pLNs (C) analyzed on day 15 post-BMT. (D), Mean ± SD absolute number of $CD4^+Foxp3^+$ cells in the spleen analyzed on day 15 post-BMT. Mice received RGI-2001 doses of 0.001-100 μg/kg on day 0. Data are the combined results from 6 independent experiments; numbers in parentheses indicate the number of animals analyzed. *P* values for treated versus untreated: **P* < .05; ‡*P* < .005.

depleted from WSCs by cell sorting before the transfusion into Balb/c recipients (Figure 5A). In the initial experiment, the effect of RGI-2001 (1 μg/kg) was investigated in mice that received TCD BMCs and $Foxp3:GFP^+$-depleted spleen cells (2.5×10^6 cells). In this group, 80% of the mice died by day 15. The mice that received unsorted WSCs (2.5×10^6 cells) or $Foxp3:GFP^+$-depleted spleen cells (2.5×10^6 cells) along with WSCs (1.25×10^6 cells) derived from wild-type C57BL/6 mice (WT WSCs) survived (Figure 5B). This finding indicates that nTregs present in the transfused donor spleen cells were required for the control of GVHD by RGI-2001. Thus, to supplement the nTregs, WT WSCs (1.25×10^6 cells) were added to $Foxp3:GFP^+$-depleted spleen cells (2.5×10^6 cells) in subsequent experiments. On day 15 post-BMT, the percentage of $CD4^+$ $Foxp3:GFP^+$ cells was investigated (Figure 5C and 5D). In mice that received 2.5×10^6 of unsorted WSCs, RGI-2001 treatment (1 μg/kg) gave rise to 9.0% ± 3.4% $CD4^+$ $Foxp3:GFP^+$ cells, an approximate 5-fold increase over the level seen in untreated mice (1.8% ± 1.0%). This level of increase was comparable to that observed with C57BL/6 wild-type WSCs, indicating comparable responsiveness of Foxp3:GFP reporter mice. In contrast, the mice that received an equivalent number of spleen cells that were depleted for $Foxp3:GFP^+$ preexisting nTregs together with 1.25×10^6 WT WSCs gave rise to only 1.6% ± 0.4% $CD4^+$ $Foxp3:GFP^+$ cells after RGI-2001 treatment, not significantly different from the level in the untreated mice (1.1% ± 0.9%). These results indicate that the increase in Tregs induced by RGI-2001 was due primarily to the expansion of preexisting $Foxp3:GFP^+$ nTregs in the transfused WSCs, with little contribution from inducible Tregs (iTregs) converted from $Foxp3:GFP^-$ $CD4^+$ cells.

Combination with a Subtherapeutic Dose of RAPA Further Improves Efficacy

RAPA has been reported to have favorable effects on Tregs [22,23]. Moreover, low-dose RAPA was found to enhance the efficacy of a Treg-inducing histone deacetylase (HDAC) inhibitor in cardiac and islet cell transplantation models [24]. Consequently, the efficacy of RGI-2001 in combination with low-dose RAPA was evaluated. The subtherapeutic low dose of RAPA was set at 0.1 mg/kg, which is lower than the

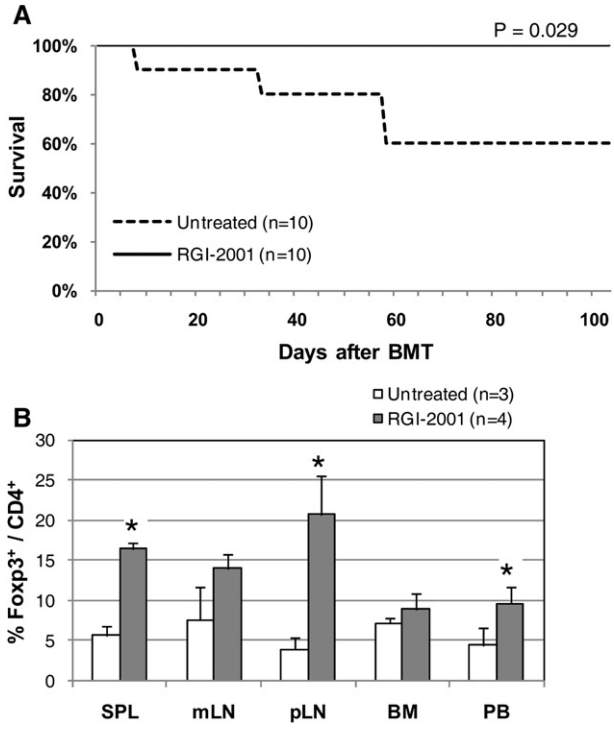

Figure 4. Efficacy of RGI-2001 in a different strain combination (FVB → Balb/c). A, Effects of RGI-2001 (1 μg/kg, day 0) on survival of lethally irradiated Balb/c mice reconstituted with 5×10^6 BMCs and 2.5×10^6 WSCs of FVB were investigated. *P* values versus untreated. B, Mean ± SD percentages of $CD4^+Foxp3^+$ cells in total $CD4^+$ cells in spleen, mLNs, pLNs, BM, and PB analyzed on day 15 post-BMT in Balb/c mice reconstituted with BMCs + WSCs derived from FVB. The results of untreated (n = 3) and RGI-200–treated (n = 4) mice are shown. *P* values for treated versus untreated: *$P < .05$.

reported optimal dose for GVHD prevention (1.5 mg/kg) [25,26]. It was confirmed that daily injections of RAPA at doses <0.5 mg/kg did not suppress the induction of cytokines or the iNKT cell expansion induced by RGI-2001 (1 μg/kg) in normal C57BL/6 mice (data not shown). In the GVHD model, the addition of low-dose RAPA to RGI-2001 reproducibly improved survival compared with either single treatment in two independent experiments (Figure 6A). The majority of mice that received the combination treatment (RGI-2001 1 μg/kg on day 0 and RAPA 0.1 mg/kg daily on days 0-14) survived for >100 days, with a statistically significant improvement over either single treatment. Clinical scores and body weights also demonstrated the greater efficacy of combination treatment in reducing the severity of acute GVHD (data not shown).

Treg levels on day 15 post-BMT were compared in the treatment groups. RGI-2001 alone and RAPA alone induced increases in the percentage of $CD4^+Foxp3^+$ cells over untreated controls in spleen (Figure 6B), mLNs (Figure 6C), and pLNs (Figure 6D). The combination of RGI-2001 and RAPA further increased Tregs to a significantly higher level in all organs compared with either single treatment. This finding was reproduced in 3 independent experiments. The absolute yields of Tregs were increased as well. In 3 experiments, the total Treg cell yield in mice that received the combination treatment was 1.7- to 3.1-fold higher than that in mice treated with RGI-2001 alone and 8.9- to 9.7-fold higher than that in those treated with RAPA alone. These increases in absolute Treg counts implied that combination treatment induced further expansion of the Treg compartment, excluding the possibility that the increased percentage of Tregs might be due to the relative enrichment of Tregs caused by RAPA-induced suppression of the conventional T cell compartment.

The effect of RAPA on Tregs was investigated using the Foxp3:GFP reporter mice (Figure 6E). In mice receiving unsorted WSCs from Foxp3:GFP mice, RAPA induced significant increases in the percentage of Tregs (8.5% ± 2.6%). The combination of RGI-2001 and RAPA further increased this percentage to 17.0% ± 3.6%. In contrast, in mice that received spleen cells depleted for $Foxp3:GFP^+$ nTregs together with WT WSCs, the levels of $Foxp3:GFP^+$ cells remained low after RAPA or RGI-2001 + RAPA treatment, indicating that the expansion of preexisting nTregs contributed to the majority of Tregs induced by RAPA or RGI-2001 + RAPA. Nonetheless, a trend (albeit marginal) toward increased $Foxp3:GFP^+$ Tregs in the mice treated with a RAPA-containing regimen was seen. In contrast to RGI-2001 single treatment (Figure 5C), the differences appeared to be statistically meaningful, suggesting the possibility that RAPA might promote the de novo conversion of naïve T cells to iTregs.

RGI-2001 Treatment Does Not Abrogate Graft-versus-Leukemia Activity

The influence of RGI-2001 on graft-versus-leukemia (GVL) activity was investigated using a well-established GVL model involving Balb/c-derived, YFP/luciferase-expressing A20 leukemia cells [19]. First, it was confirmed that RGI-2001 treatment did not affect the growth of A20-*luc/yfp* cells in vivo. In lethally irradiated Balb/c mice undergoing transplantation with syngeneic BMC and A20-*luc/yfp* cells, a distinct population of $B220^{hi}$/YFP-Luc^+ leukemia cells was noted in the spleen on day 26 (Figure 7A). The levels of leukemia cell growth were comparable in both RGI-2001–treated and untreated mice. The growth of leukemia cells was then investigated in mice reconstituted with allogeneic C57BL/6 TCD BMCs. In mice undergoing transplantation with C57BL/6 TCD BMCs without WSCs, growth of leukemia cells was macroscopically evident in the liver and spleen. FACS analysis of cells from spleen and BM on day 32 revealed the $B220^{hi}$/YFP-Luc^+

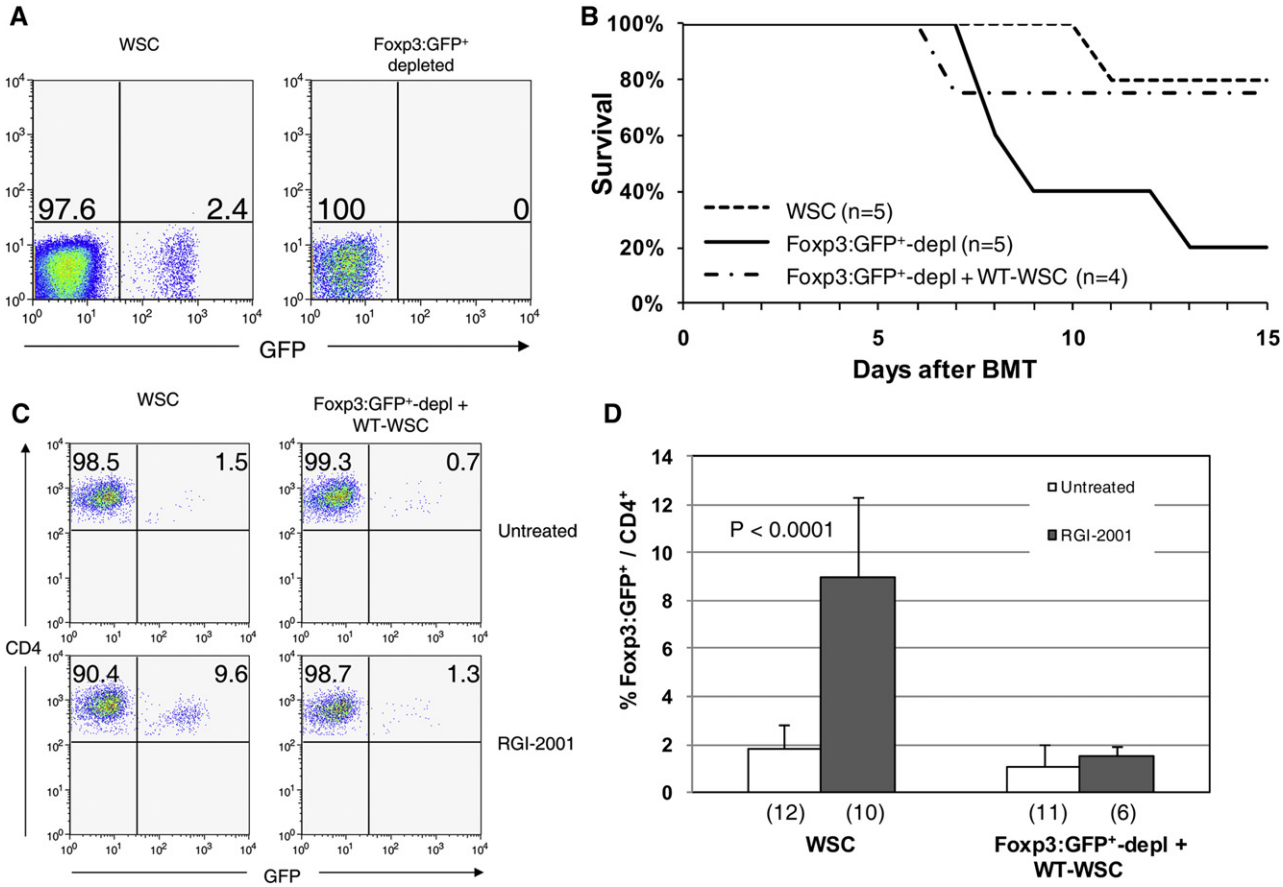

Figure 5. Expansion of donor-derived GFP$^+$ nTregs by RGI-2001 treatment. The origin of CD4$^+$Foxp3$^+$ cells induced by RGI-2001 after BMT was investigated using Foxp3:GFP reporter mice as donors. A, Representative dot plots showing WSCs and sorted Foxp3:GFP$^+$-depleted spleen cells. The cell sorting strategy routinely yielded >99% purity in Foxp3:GFP$^+$-depleted spleen cells. B, Survival of Balb/c recipients that received various spleen cell preparations: 2.5 $\times$ 10^6 WSCs containing Foxp3:GFP$^+$ nTregs (WSC), 2.5 $\times$ 10^6 spleen cells depleted of Foxp3:GFP$^+$ nTregs (Foxp3:GFP$^+$-depl), or 2.5 $\times$ 10^6 spleen cells depleted of Foxp3:GFP$^+$ nTregs and 1.25 $\times$ 10^6 wild-type C57BL/6 WSCs (Foxp3:GFP$^+$-depl + WT WSC). Mice were treated with RGI-2001 (1 μg/kg, day 0). By day 15, 80% of the mice that had received Foxp3:GFP$^+$-depl died. The *P* value for Foxp3:GFP$^+$-depl versus WSC was 0.0564 (log-rank test). The mice that received WSC (n = 5), Foxp3:GFP$^+$-depl (n = 5), or Foxp3:GFP$^+$-depl + WT WSC (n = 4), but not RGI-2001, all survived until day 15. C and D, Recovery of CD4$^+$Foxp3:GFP$^+$ Tregs in untreated and RGI-2001–treated mice was investigated on day 15 post-BMT. C, Representative dot plots showing GFP$^+$ cells in CD4$^+$ T cells in mice that received WSC or Foxp3:GFP$^+$-depl + WT WSC, with or without RGI-2001 treatment. D, Mean ± SD percentages of CD4$^+$Foxp3:GFP$^+$ cells in total CD4$^+$ cells. Data are combined results from 4 independent experiments; numbers in parentheses indicate the number of animals analyzed. *P* values versus untreated. Injected WSCs contained on average 6.0 $\times$ 10^4 Foxp3:GFP$^+$ cells. On day 15, 68.2 ± 27.3 $\times$ 10^4 Foxp3:GFP$^+$ cells were recovered from the spleens in the treated group (n = 8), indicating a robust expansion (11.4-fold) of Foxp3:GFP$^+$ nTregs. The spleens in the untreated group contained 4.2 ± 2.4 $\times$ 10^4 Foxp3:GFP$^+$ cells (n = 8).

leukemia cell population in these mice, whereas no leukemia cells were detected in the mice that received C57BL/6 WSCs + A20-*luc/yfp* with or without RGI-2001 (Figure 7B). An experiment following survival showed that the mice that did not receive the WSCs did not develop GVHD symptoms but began dying on day 24, and all died of leukemia by day 39 (Figure 7C). The mice that received only WSCs all died by day 39 due to acute GVHD. The mice receiving WSCs + A20-*luc/yfp* developed clinical signs of GVHD. The survival curve of this group was very similar to that of the WSC-only group, indicating acute GVHD as the cause of death in this group. In contrast, the mice receiving WSCs + A20-*luc/yfp* + RGI-2001 survived longer, although they also developed clinical GVHD. In this group, 50% of the mice died of GVHD, but the remaining 50% recovered from GVHD and survived up to termination of the experiment on day 68, which was in the range anticipated for the mice treated with RGI-2001, as noted earlier (1 μg/kg; Figure 1B). At study termination on day 68, no YFP-Luc$^+$ leukemia cells were detected in these mice. Similar survival results were reproducibly observed in 3 independent experiments.

The levels of CD4$^+$Foxp3$^+$ cells in mice that received A20-*luc/yfp* cells were then analyzed on day 15 post-BMT. The mice receiving WSCs + A20-*luc/yfp* + RGI-2001 (n = 4) had 19.3% ± 6.5% CD4$^+$ Foxp3$^+$ cells in the spleen, a level comparable to that seen in historical controls receiving WSCs + RGI-2001 (14.8% ± 7.6%; Figure 2B). These results indicate that the presence of leukemia cells did not influence the Treg expansion by RGI-2001, and that Tregs induced by RGI-2001 in the presence of

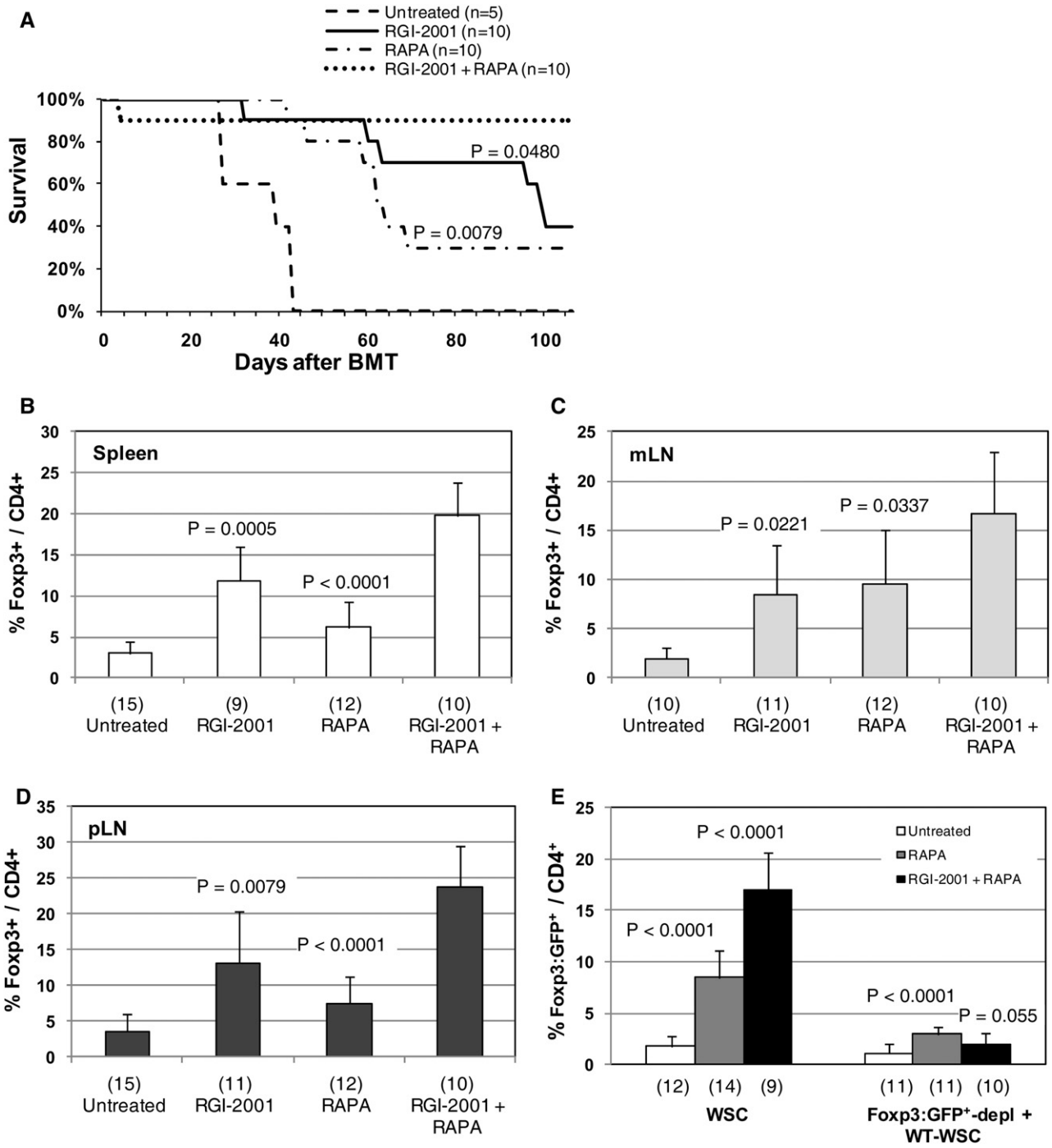

Figure 6. Enhanced efficacy and Treg induction by RGI-2001 in combination with a subtherapeutic dose of RAPA. Effects of combination treatment with RGI-2001 (1 μg/kg, day 0) and a subtherapeutic dose of RAPA (0.1 mg/kg i.p. daily on days 0-14) on survival and Treg induction were evaluated. A, Survival curves of mice that received RGI-2001 alone, RAPA alone, or RGI-2001 + RAPA. Results of two experiments were combined. *P* values comparing either RGI-2001 or RAPA single-treatment group versus RGI-2001 + RAPA treatment group. B, C, and D, Mean ± SD percentage of $CD4^+Foxp3^+$ cells in total $CD4^+$ cells in the spleen (B), mLNs (C), and pLNs (D) analyzed on day 15 post-BMT. Results are from 3 independent experiments; numbers in parentheses indicate the number of animals analyzed. Historical untreated control data are included as a reference. *P* values comparing either RGI-2001 or RAPA single treatment group versus RGI-2001 + RAPA treatment group. E, The origin of $CD4^+Foxp3^+$ cells induced by the RAPA-containing regimen was investigated using Foxp3:GFP reporter mice as donors. Mice received WSCs or $Foxp3:GFP^+$-depleted spleen cells and WT WSCs, as described in Figure 5, and were treated with RAPA or RGI-2001 + RAPA or were untreated. The mean ± SD percentage of $CD4^+Foxp3:GFP^+$ cells in total $CD4^+$ cells in the spleen analyzed on day 15 post-BMT is shown. Data are combined results from 4 independent experiments; numbers in parentheses indicate the number of animals analyzed. *P* values are versus untreated.

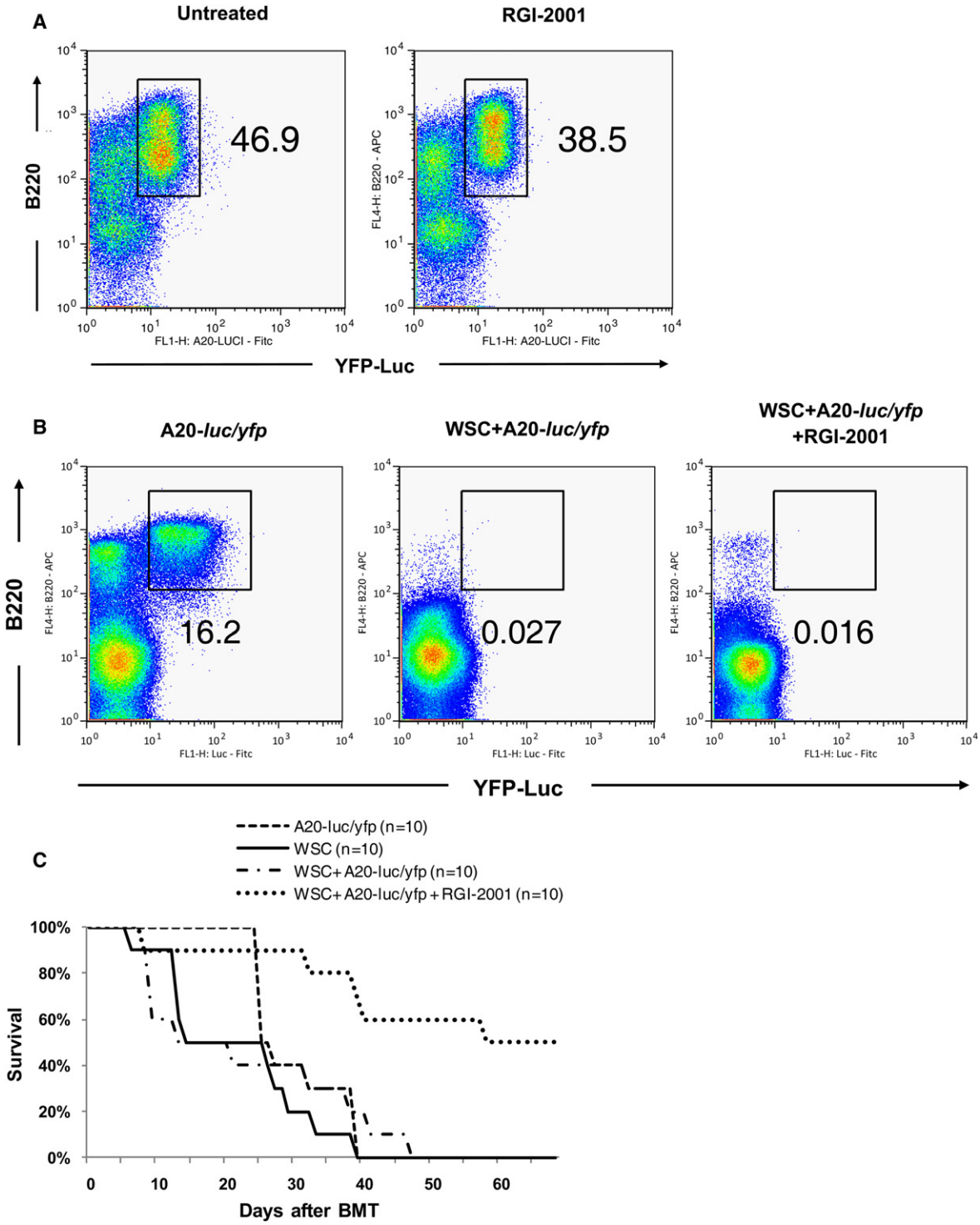

Figure 7. Effects of RGI-2001 on GVL activity were investigated using a GVL model involving Balb/c-derived, YFP/luciferase-expressing A20 leukemia cells (A20-*luc/yfp*). A, Growth of A20-*luc/yfp* in a syngeneic BMT model with and without RGI-2001 treatment. Lethally irradiated Balb/c mice received syngeneic BMCs (5×10^6) and A20-*luc/yfp* (5×10^4) cells with or without RGI-2001 treatment (1 μg/kg, day 0) and were analyzed for leukemia cell growth on day 26 (n = 3 for each group). Representative FACS plots of the spleen illustrate a distinct population of $B220^{hi}$/YFP-Luc^+ leukemia cells in both untreated and RGI-2001–treated mice. RGI-2001 did not suppress the growth of A20-*luc/yfp*. B, Growth of A20-*luc/yfp* in a GVHD model with and without RGI-2001 treatment. Lethally irradiated Balb/c mice received C57BL/6 TCD BMCs (5×10^6) along with A20-*luc/yfp* (5×10^4), C57BL/6 WSCs (2.5×10^6) + A20-*luc/yfp* (5×10^4), or C57BL/6 WSCs (2.5×10^6) + A20-*luc/yfp* (5×10^4) + RGI-2001 (1 μg/kg) and were analyzed on day 32 (n = 3 for each group). Representative FACS plots of the BM demonstrate the growth of the $B220^{hi}$/YFP-Luc^+ leukemia cells in mice that received A20-*luc/yfp* without C57BL/6 WSCs. No leukemia cells were detected in mice that received C57BL/6 WSCs + A20-*luc/yfp* with or without RGI-2001. C, Survival of mice that received A20-*luc/yfp*. Lethally irradiated Balb/c mice received C57BL/6 TCD BMCs along with A20-*luc/yfp*, C57BL/6 WSCs, C57BL/6 WSCs + A20-*luc/yfp*, or C57BL/6 WSCs + A20-*luc/yfp* + RGI-2001, with the same protocol as in B. Results of a representative experiment out of 4 experiments are shown.

leukemia cells did not inhibit eradication of leukemia cells. Taken together, these results demonstrate that RGI-2001 suppressed acute GVHD without compromising the GVL effect.

DISCUSSION

Our results indicate that a liposomal formulation of KRN7000 (RGI-2001) can prevent acute GVHD by expanding Tregs in vivo. In a fully MHC-mismatched acute GVHD murine model, a single i.v. injection of RGI-2001 at the time of BMT demonstrated significant efficacy in prolonging the survival of mice that were otherwise acutely fatal. In mice treated with RGI-2001, host alloantigen-specific tolerance was introduced early after BMT. The immune response against third-party alloantigens and leukemia cells was not compromised. RGI-2001 treatment induced significant increases in donor-derived $CD4^+Foxp3^+$ nTregs in the secondary lymphoid organs including spleen, mLNs, and pLNs. The increase in nTregs was noted in the spleen as early as day 6 post-BMT. The survival of mice that received nTreg-depleted donor cells was not improved by RGI-2001 treatment. Based on these findings, effective Treg induction by RGI-2001 can be considered the key mechanism of action underlying the reduced mortality and prolonged survival in the RGI-2001–treated mice.

Collectively, our results demonstrate the efficacy of pharmacologically induced nTregs in preventing acute GVHD without abrogating the GVL effect. The finding is in line with the pivotal role of donor-derived Tregs in controlling GVHD reported in both experimental and clinical studies [27-32]. In mice, adoptive transfer of donor-derived $CD4^+CD25^+$ Tregs was shown to inhibit GVHD while maintaining GVL [29-30]. Likewise, it was recently reported that adoptive immunotherapy with donor-derived $CD4^+CD25^+$ Tregs prevented GVHD in patients who received haploidentical transplants for the treatment of high-risk hematologic malignancies [33]. Our findings indicate the efficacy of Tregs pharmacologically induced in vivo, corroborating the earlier findings generated using the adoptive transfer of Tregs.

RGI-2001 treatment significantly enhanced the expansion of donor-derived $CD4^+Foxp3^+$ Tregs early after BMT. Using the Foxp3:GFP reporter mice as donors, we clearly showed that these Tregs were derived from existing nTregs within the transfused donor spleen cells. It is conceivable that RGI-2001 promoted the rapid expansion of nTregs with pre-endowed antigen specificity to host antigens in an allogeneic host environment. The marked suppression seen in MLRs stimulated with host cells indicates that the expanded nTregs were functional and facilitated host alloantigen-specific immune suppression. Our results are in accordance with those of a previous study that directly compared the efficacy of GVHD prevention of nTregs and iTregs using Foxp3:GFP reporter mice [34,35]. Koenecke et al. [34] reported that adaptively transferred FACS-sorted nTregs significantly improved survival and morbidity, whereas iTregs, converted from $Foxp3:GFP^-$ CD4 T cells in vitro, rapidly lost the Treg features and failed to prevent GVHD. In contrast, another study found that iTregs converted from effector T cells in vivo by the hypomethylating agent azacitidine mitigated GVHD while preserving the GVL effect [36]. In that study, however, the suppressor function of these cells was mediated through the modulation of Foxp3 target genes by azacitidine, independent of Foxp3 expression [36].

In the present study, significant increases in Tregs in mLNs and pLNs were noted early (by day 15) after BMT, and significant increases in Tregs in the spleen were observed as early as day 6 post-BMT. Furthermore, delayed treatment with RGI-2001 resulted in a loss of efficacy, reinforcing the importance of early activity of Tregs in GVHD prevention at the site of GVHD initiation. Secondary lymphoid organs, particularly lymph nodes proximal to GVHD target organs, are considered the sites for GVHD induction [37,38]. Thus, Tregs may efficiently execute their suppressive activity in these sites, where host antigen-presenting cells actively stimulate donor-derived alloreactive T cells. A study using whole-body bioluminescence imaging elegantly demonstrated that adoptively transferred Tregs initially localize and expand in the secondary lymphoid organs, such as mLNs, pLNs, and spleen [32]. Other studies found that adoptively transferred Tregs, particularly those expressing homing molecules to the secondary lymphoid organs, such as CD62L (L-selectin), ameliorate GVHD by interfering with the activation, expansion, and migration of GVHD effector T cells in these sites [39,40]. Those studies demonstrated that Treg activity early after BMT (before the onset of the effector phase) is essential for GVHD prevention [32,39]. Taken together, these previous findings and our present results indicate that spatially and temporally coordinated Treg activity is the key for effective prevention of GVHD. Of note, Treg levels in mLNs and pLNs decreased by day 30 post-BMT. Investigating whether RGI-2001 treatment leads to increased Treg levels in GVHD target organs, such as the gut and liver, by this time point would be of interest.

The action of RGI-2001 is initiated through the interaction of KRN7000 and iNKT cells. The role of NKT cells in GVHD prevention has been demonstrated through the establishment of nonmyeloablative total lymphoid irradiation (TLI) conditioning of the host [41-45]. Fractionated irradiation of the lymphoid

tissues results in marked enrichment of NKT cells in the host lymphoid organs, due primarily to the more profound depletion of non-NKT cell populations [41]. Mice conditioned with a TLI regimen are protected from GVHD, which is lethal for hosts conditioned with total body irradiation. GVHD protection by TLI conditioning is lost when donor $CD4^+CD25^+$ Tregs are depleted before transplantation [43], and host NKT- and IL-4–dependent expansion of donor-derived Tregs provides a mechanism to regulate GVHD in this regimen [45]. Thus, GVHD protection afforded by RGI-2001 and TLI conditioning might possibly share a common underlying mechanism. Both host- and donor-derived NKT cells have been reported to prevent GVHD [46]. Future studies using NKT-lacking knockout mice, such as $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice, will help identify the origin of NKT cells involved in GVHD suppression by RGI-2001. Although the mechanisms downstream of NKT cells leading to Treg induction are not clearly understood, we recently found that RGI-2001 preferentially induces the expansion of tolerogenic plasmacytoid DCs that precedes Treg expansion (data not shown).

The results of our experiments using the Foxp3:GFP reporter mice clearly demonstrate that the majority of Tregs induced by RGI-2001 and/or RAPA were derived from pre-existing nTregs. However, in the mice treated with a RAPA-containing regimen, marginal but statistically meaningful increases in Tregs were noted in those mice transfused with $Foxp3:GFP^+$-depleted spleen cells, suggesting a minor contribution of de novo converted iTregs induced by RAPA treatment. Accumulated evidence in recent years indicates that RAPA exerts favorable effects on Tregs [22,23]. RAPA has been reported to preferentially inhibit the proliferation of conventional T cells while sparing Tregs [26,47], to induce selective expansion of murine [48] and human [6,49] naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs in vitro, and to induce Foxp3 expression and regulatory functions in conventional $CD4^+$ T cells in vitro [49,50]. Our results indicate that RAPA promotes expansion of nTregs and may also promote, to a lesser extent, de novo induction of iTregs in vivo in a lymphopenic allogeneic condition.

In the present study, the efficacy of RGI-2001 in attenuating the course of acute GVHD was seen at doses of 0.1-100 μg/kg. This dose range correlates well with the biological activity measured by cytokine induction (≥0.01 μg/kg; Figure S1A-C) and Treg expansion (≥0.01 μg/kg; Figure 3). Dose-related protection from GVHD lethality by RGI-2001 was observed at doses of 0.001-0.1 μg/kg, with no efficacy at 0.001 μg/kg, limited efficacy at 0.01 μg/kg, and significant efficacy at 0.1 μg/kg. However, within the effective dose range (0.1-100 μg/kg), no clear dose-related improvement in survival was noted (Figure 1A and B) despite the dose-dependent increases in Tregs (Figure 3D). It is possible that the magnitude of increase in Tregs within the effective dose range might not be sufficient to translate into a robust improvement in survival. The changes in the Treg:T conventional T cell ratio, which is considered to affect the efficacy of Tregs [28], was modest within the effective dose range. The increase in the $CD4^+Foxp3^+$ Treg:$CD4^+Foxp3^-$ T conventional T cell ratio in the spleen was about 2-fold, from 1:9 at 0.1 μg/kg (minimum effective dose) to 1:4 at 100 μg/kg. Likewise, the increase in absolute Treg numbers was ~5-fold at the 100-μg/kg dose compared with the 0.1-μg/kg dose. However, when combined with a subtherapeutic dose of RAPA, survival improved significantly. Although this survival improvement was associated with synergistic increases in Tregs, additional effects of RAPA on other cell types, such as suppression of effector cells, might possibly be attributable to the improved efficacy.

Contradictory to our findings, a recent study found that KRN7000 induced hyperacute GVHD in mice reconstituted with hematopoietic stem cells derived from the spleen of granulocyte-colony stimulating factor–treated mice [51]. In the present study, no acceleration of GVHD was observed at any of the doses evaluated. Differences in the hematopoietic stem cell source may explain the difference in outcomes; Kuns et al. [51] reported a lower incidence of hyperacute GVHD with the use of BMCs instead of spleen cells from granulocyte-colony stimulating factor–treated mice. Furthermore, no hyperacute GVHD was reported in previous studies evaluating the effect of KRN7000 in GVHD using BMCs [52]. Further studies are needed to identify the precise mechanisms involved in these contradictory effects.

A promising result reported from a recent clinical study evaluating the efficacy in GVHD prevention of adoptive immunotherapy with freshly isolated donor-derived Tregs [33] corroborates the pivotal role of Tregs in GVHD control in humans. Considering the lack of efficacy of in vitro expanded Tregs, either iTregs [34,35], or nTregs [53], along with the paucity of therapeutic nTregs in PB, drugs that can pharmacologically induce nTregs in vivo would provide a significant benefit. Several compounds in addition to RGI-2001 have been reported to induce Tregs in vivo through different mechanisms. The HDAC inhibitor trichostatin A increases Foxp3 gene expression as well as the production and suppressive function of Foxp3-dependent Tregs through inhibition of HDAC9 [24]. An agonist of the aryl hydrocarbon receptor, VAF347, induces long-term active tolerance to alloantigens through a direct effect on Treg survival and function, as well as through DC-mediated induction of Tregs [54]. The hypomethylating agent azacitidine reportedly converts effector T cells into $Foxp3^+$ iTregs and induces suppressor function

through epigenetic modulation of Foxp3 and its target genes [36]. Induction and maintenance of antigen-specific tolerance by compounds that exploit intrinsic immunomodulatory mechanisms may provide significant clinical benefits to patients undergoing allogeneic hematopoietic stem cell transplantation, as well as those undergoing solid organ transplantation.

ACKNOWLEDGMENTS

We thank Dr. Teikichi Aoyagi, Dr. Lisa Hernandez, and Haruhiko Morita for valuable discussions, as well as Dr. James Bakke of SRI International, Annabelle Teng from the Riken Institute, and Tuyen Trinh from REGiMMUNE for technical assistance. This study was supported by research funding from REGiMMUNE and a grant from the Japanese National Institute of Biomedical Innovation.

Financial disclosure: Omar Duramad, Amy Laysang, and Jun Li are employees of REGiMMUNE. Yasuyuki Ishii is the founder of the company, and Reiko Namikawa is a consultant. Yasuyuki Ishii and Reiko Namikawa have ownership interests in REGiMMUNE.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbmt.2010.11.022

REFERENCES

1. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self-tolerance and autoimmunity. *Nat Immunol*. 2009; 11:7-13.
2. Sagoo P, Lombardi G, Lechler RI. Regulatory T cells as therapeutic cells. *Curr Opin Organ Transplant*. 2008;13:645-653.
3. Long E, Wood KJ. Regulatory T cells in transplantation: transferring mouse studies to the clinic. *Transplantation*. 2009;88: 1050-1056.
4. Socie G, Blazar BR. Acute graft-versus-host disease: from the bench to the bedside. *Blood*. 2009;114:4327-4336.
5. Hoffmann P, Eder R, Kunz-Schughart LA, et al. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood*. 2004;104:895-903.
6. Battaglia M, Stabilini A, Migliavacca B, et al. Rapamycin promotes expansion of functional $CD4^+CD25^+FOXP3^+$ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol*. 2006;177:8338-8347.
7. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009; 30:656-665.
8. Shatry A, Chirinos J, Gorin MA, et al. Targeting Treg cells in situ: emerging expansion strategies for (CD4(+)CD25(+)) regulatory T cells. *Biol Blood Marrow Transplant*. 2009;15: 1239-1243.
9. Natori T, Koezuka Y, Higa T. Agelasphins, novel α-galactosylceramides from the marine sponge *Agelas mauritianus*. *Tetrahedron Lett*. 1993;34:5591-5592.
10. Kawano T, Cui J, Koezuka Y, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*. 1997;278:1626-1629.
11. Brossay L, Chioda M, Burdin N, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med*. 1998;188:1521-1528.
12. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol*. 2004;22:817-890.
13. Moody DB, Zajonc DM, Wilson IA. Anatomy of CD1–lipid antigen complexes. *Nat Rev Immunol*. 2005;5:387-399.
14. Taniguchi M, Harada M, Kojo S, et al. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol*. 2003;21:483-513.
15. Tamura Y, Teng A, Nozawa R, et al. Characterization of the immature dendritic cells and cytotoxic cells both expanded after activation of invariant NKT cells with alpha-galactosylceramide in vivo. *Biochem Biophys Res Commun*. 2008;369:485-492.
16. Ishii Y, Nozawa R, Takamoto-Matsui Y, et al. Alpha-galactosylceramide–driven immunotherapy for allergy. *Front Biosci*. 2008; 13:6214-6228.
17. Haribhai D, Lin W, Relland LM, et al. Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol*. 2007;178:2961-2972.
18. Matsuda JL, Naidenko OV, Gapin L, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med*. 2000;192:741-754.
19. Edinger M, Cao YA, Verneris MR, et al. Revealing lymphoma growth and the efficacy of immune cell therapies using in vivo bioluminescence imaging. *Blood*. 2003;101:640-648.
20. Stull RA, Hyun WC, Pallavicini MG. Simultaneous flow cytometric analyses of enhanced green and yellow fluorescent proteins and cell surface antigens in doubly transduced immature hematopoietic cell populations. *Cytometry*. 2000;40:126-134.
21. Van Kaer L. alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat Rev Immunol*. 2005;5: 31-42.
22. Demirkiran A, Hendrikx TK, Baan CC, et al. Impact of immunosuppressive drugs on $CD4^+CD25^+FOXP3^+$ regulatory T cells: does in vitro evidence translate to the clinical setting? *Transplantation*. 2008;85:783-789.
23. Zeiser R, Negrin RS. Interleukin-2 receptor downstream events in regulatory T cells: implications for the choice of immunosuppressive drug therapy. *Cell Cycle*. 2008;7:458-462.
24. Tao R, de Zoeten EF, Ozkaynak E, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med*. 2007;13:1299-1307.
25. Antin JH, Kim HT, Cutler C, et al. Sirolimus, tacrolimus, and low-dose methotrexate for graft-versus-host disease prophylaxis in mismatched related donor or unrelated donor transplantation. *Blood*. 2003;102:1601-1605.
26. Zeiser R, Leveson-Gower DB, Zambricki EA, et al. Differential impact of mammalian target of rapamycin inhibition on $CD4^+CD25^+Foxp3^+$ regulatory T cells compared with conventional $CD4^+$ T cells. *Blood*. 2008;111:453-462.
27. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood*. 2002;99: 3493-3499.
28. Hoffmann P, Ermann J, Edinger M, et al. Donor-type CD4(+) CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. 2002;196:389-399.
29. Edinger M, Hoffmann P, Ermann J, et al. $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med*. 2003;9:1144-1150.
30. Trenado A, Charlotte F, Fisson S, et al. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest*. 2003;112:1688-1696.

31. Joffre O, Gorsse N, Romagnoli P, et al. Induction of antigen-specific tolerance to bone marrow allografts with $CD4^+CD25^+$ T lymphocytes. *Blood*. 2004;103:4216-4221.
32. Nguyen VH, Zeiser R, Dasilva DL, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood*. 2007;109:2649-2656.
33. Di Ianni M, Falzetti F, Carotti A, et al. Adoptive immunotherapy with Tregs prevents GVHD and favours immune reconstitution after HLA-haploidentical transplants for hematological malignancies. *Blood (ASH Annual Meeting Abstracts)*. 2009; 114:4.
34. Koenecke C, Czeloth N, Bubke A, et al. Alloantigen-specific de novo–induced $Foxp3^+$ Treg revert in vivo and do not protect from experimental GVHD. *Eur J Immunol*. 2009;39:3091-3096.
35. Edinger M. Regulatory T cells for the prevention of graft-versus-host disease: professionals defeat amateurs. *Eur J Immunol*. 2009;39:2966-2968.
36. Choi J, Ritchey J, Prior JL, et al. In vivo administration of hypomethylating agents mitigate graft-versus-host disease without sacrificing graft-versus-leukemia. *Blood*. 2010;116:129-139.
37. Ferrara JL, Reddy P. Pathophysiology of graft-versus-host disease. *Semin Hematol*. 2006;43:3-10.
38. Sun Y, Tawara I, Toubai T, et al. Pathophysiology of acute graft-versus-host disease: recent advances. *Transl Res*. 2007;150: 197-214.
39. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, et al. L-Selectin(hi) but not the L-selectin(lo) $CD4^+25^+$ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood*. 2004;104:3804-3812.
40. Ermann J, Hoffmann P, Edinger M, et al. Only the $CD62L^+$ subpopulation of $CD4^+CD25^+$ regulatory T cells protects from lethal acute GVHD. *Blood*. 2005;105:2220-2226.
41. Lan F, Zeng D, Higuchi M, et al. Predominance of $NK1.1^+$TCR alpha $beta^+$ or $DX5^+$TCR alpha $beta^+$ T cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "natural suppressor" cells. *J Immunol*. 2001;167:2087-2096.
42. Lan F, Zeng D, Higuchi M, et al. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biol Blood Marrow Transplant*. 2003;9:355-363.
43. Pillai AB, George TI, Dutt S, et al. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol*. 2007;178: 6242-6251.
44. Liu YP, Li Z, Nador RG, et al. Simultaneous protection against allograft rejection and graft-versus-host disease after total lymphoid irradiation: role of natural killer T cells. *Transplantation*. 2008;85:607-614.
45. Pillai AB, George TI, Dutt S, et al. Host natural killer T cells induce an IL-4 dependent expansion of donor $CD4^+CD25^+$ $Foxp3^+$ Tregs that protects against graft-versus-host disease. *Blood*. 2009;113:4458-4467.
46. Kohrt HE, Pillai AB, Lowsky R, et al. NKT cells, Treg, and their interactions in bone marrow transplantation. *Eur J Immunol*. 2010;40:1862-1869.
47. Keever-Taylor CA, Browning MB, Johnson BD, et al. Rapamycin enriches for CD4(+) CD25(+) CD27(+) Foxp3(+) regulatory T cells in ex vivo-expanded CD25-enriched products from healthy donors and patients with multiple sclerosis. *Cytotherapy*. 2007;9:144-157.
48. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood*. 2005; 105:4743-4748.
49. Haxhinasto S, Mathis D, Benoist C. The AKT–mTOR axis regulates de novo differentiation of $CD4^+Foxp3^+$ cells. *J Exp Med*. 2008;205:565-574.
50. Valmori D, Tosello V, Souleimanian NE, et al. Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated $CD4^+$ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional $CD4^+$ T cells. *J Immunol*. 2006;177:944-949.
51. Kuns RD, Morris ES, Macdonald KP, et al. Invariant natural killer T cell–natural killer cell interactions dictate transplantation outcome after alpha-galactosylceramide administration. *Blood*. 2009;113:5999-6010.
52. Morecki S, Panigrahi S, Pizov G, et al. Effect of KRN7000 on induced graft-vs-host disease. *Exp Hematol*. 2004;32:630-637.
53. Brunstein C, Hippen KL, McKenna DH, et al. Adoptive transfer of umbilical cord blood (UCB)-derived regulatory T cells (Tregs) to recipients of nonmyeloablative unrelated double UCB transplantation. *Blood (ASH Annual Meeting Abstracts)*. 2009;114:513.
54. Hauben E, Gregori S, Draghici E, et al. Activation of the aryl hydrocarbon receptor promotes allograft-specific tolerance through direct and dendritic cell-mediated effects on regulatory T cells. *Blood*. 2008;112:1214-1222.